Journal of Forensic and Legal Medicine 20 (2013) 334–338

Contents lists available at SciVerse ScienceDirect

Journal of Forensic and Legal Medicine

journal homepage: www.elsevier.com/locate/jflm

Short report

Mitochondrial 16S ribosomal RNA gene for forensic identification of crocodile species

K. Naga Jogayya MSc, Senior Research Fellow [a], P.R. Meganathan PhD, Post Doctoral Fellow [b], Bhawna Dubey PhD, Research Associate [c], I. Haque PhD, Assistant Director [a,*]

[a] Biology Divison, Central Forensic Science Laboratory, 30, Gorachand Road, Park Circus, Kolkata 700014, West Bengal, India
[b] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA
[c] Molecular Genetics Laboratory, Center for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

ARTICLE INFO

Article history:
Received 27 September 2011
Received in revised form
15 July 2012
Accepted 7 September 2012
Available online 26 October 2012

Keywords:
Crocodylia
Forensic identification
16S rRNA
Conservation
Phylogeny

ABSTRACT

All crocodilians are under various threats due to over exploitation and these species have been listed in Appendix I or II of CITES. Lack of molecular techniques for the forensic identification of confiscated samples makes it difficult to enforce the law. Therefore, we herein present a molecular method developed on the basis on 16S rRNA gene of mitochondrial DNA for identification of crocodile species. We have developed a set of 16S rRNA primers for PCR based identification of crocodilian species. These novel primers amplify partial 16S rRNA sequences of six crocodile species which can be later combined to obtain a larger region (1290 bp) of 16S rRNA gene. This 16S rRNA gene could be used as an effective tool for forensic authentication of crocodiles. The described primers hold great promise in forensic identification of crocodile species, which can aid in the effective enforcement of law and conservation of these species.

1. Introduction

Crocodiles are the largest species among the members of class Reptilia in vertebrates and are one of the last living links to the era when dinosaurs lived. Until the early 1950s, these species were numerous in the tropics of Africa, the Americas, Asia and Australia. In the recent years many of crocodilian species are considered endangered due to their habitat destruction and illegal poaching for their lucrative products.[34,35] According to Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora, crocodilian species were grouped in Appendix I and II. A significant illegal trade of crocodilians has engrossed a lot of attention and therefore the species deserve priority for conservation. In order to conserve these antiquities many countries have developed legal policies for prohibition of poaching and hunting.[28] However, illegal trade still exists in many parts of world which appears to be a challenging task to proscribe. Moreover, the biological materials are often degraded quality, which do not lead to conclusive identification through conventional methods.[6,39] In this perspective, development of novel methods for the identification of these species is inevitable towards their conservation.

Mitochondrial (mt) DNA is valuable tool for species identification from highly degraded samples.[1,11] Many of universal mt DNA markers such as cytochrome *b*,[18,38] 12S rRNA,[17] 16S rRNA,[14] and noncoding D-Loop region[28,40] have shown their potential targets for animal species identifications. Within crocodiles a single mtDNA marker i.e. cytochrome *b* (cyt *b*) has been studied as a potential source for species identification.[21–23,25,27] However, the efficiency of other regions for forensic identification of crocodiles has not been evaluated and thus these markers also need to be studied in forensic analyses.

The 16S rRNA gene, which encodes the mitochondrial large ribosomal subunit (mt LSU) in animals, has been employed extensively to explore phylogenetic relationships in anthropods at most phylogenetic levels,[8] familial level[2] and the genus level and below.[5,7,15] The wide range in utility of 16S at various taxonomic levels suggests that it could be useful marker for forensic identifications as well.

In this paper, we develop novel primer sets to amplify partial 16S ribosomal RNA region for discriminating the crocodile species. The primers were optimized and checked for their efficiency in six species i.e. *Crocodylus palustris*, *Crocodylus porosus*, *Gavialis*

* Corresponding author. Tel.: +91 33 2284 1638; fax: +91 33 2284 9442.
E-mail address: haque_cfslk@yahoo.co.in (I. Haque).

1752-928X/$ – see front matter © 2012 Elsevier Ltd and Faculty of Forensic and Legal Medicine. All rights reserved.
http://dx.doi.org/10.1016/j.jflm.2012.09.018

gangeticus, *Crocodylus niloticus*, *Crocodylus siamensis* and *Crocodylus crocodilus* which represent the three existing families of Crocodylia.

2. Materials and methods

2.1. Sample collection and DNA extraction

Authenticated biological samples were obtained from Madras Crocodile Bank Trust (MCBT), Centre for Herpetology, Mamallapuram, Tamilnadu, India, National Chambal Sanctuary Project, Agra, Uttar Pradesh, India, Nehru Zoological Park, Hyderabad, Andhra Pradesh, India, under the consent of Ministry of Environment and Forests, Government of India, New Delhi. Whole blood samples of *C. palustris*, *C. porosus*, *Crocodylus siamensis*, *C. niloticus*, *Caiman crocodilus*, and *G. gangeticus* were provided by Madras Crocodile Bank Trust (MCBT), Tamilnadu, India and 15 dead gharial samples (fresh tissue, putrified tissue and bone) were provided by National Chambal sanctuary, Utter Pradesh, India. Sample numbers and breed of each species are listed in Table 1. Total Genomic DNA extraction from blood samples were carried out by standard phenol-chloroform procedure[33] and cleaned using Microcon 100 centrifugal filter column (Millipore). DNA extraction from tissue samples was performed using Qia tissue DNA extraction kit (Qiagen, Valencia, A) as per the manufacturer's guidelines, The DNA extracts were adjusted to 50 μL in volume with TE^{-4} buffer and kept at −80 °C until use. The quality and quantity of all DNA samples isolated from blood (100–200 ng) and tissue (50 ng) was checked in 1% agarose gel.

2.2. Primer design and PCR amplification

In order to construct these primers, the complete mt DNA sequence of available crocodiles species were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) database (Table 2) and aligned using Clustal W program[36] and MEGA 5.[37] Two of the highly conserved regions in 16S rRNA gene, in all crocodiles species were chosen and the primers, Set I: 16SFI: 5′-AAA GCA TTC TGC CTA CAC CTG AAA-3′; 16SRI: 5′-TTG TGT TGG CTG CTT TAA GGC CTA -3′ and Set II: 16SFII: 5′- TGC TTG GGA ACA GAA TAT AAG TTC-3′; 16SRII: 5′-CTG CTT TTG CAC AGG GAG ATC AAT TTC-3′ were designed.

PCR reactions were carried out using a total reaction volume of 20 μl containing 2.0 μL of dNTPs (2.5 mM each) (MBI Fermentas, Glen Burnie, MD), 2.0 μL of 10X buffer (Containing 200 mM Tris–HCl, pH 8.4, and 500 mM KCl) (Invitrogen Life Technologies), 0.6 μL of Exprime *Taq* DNA polymerase (5 U/μL) (Genet Bio), and 1.0 μL of genomic DNA (100 pg). Sterile water was added to make up the volume to 20 μl. All the PCR reactions were carried out on GenAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following cycle conditions: 94 °C for 5 min of initial denaturation followed by 30 cycles of: denaturation at 95 °C for 30 s; annealing at 63 °C (Set I primers), 66 °C (Set II primers) for 30 s, extension at 72 °C for 30 s and amplification ended with a 7 min final extension step followed by a 4 °C hold. The amplicons were checked in 2% agarose gel along with a negative control.

Table 1
List of animals investigated in this study (*N* – No. of individual samples).

Species	*N* (total)	Breed	ID number
Gavialis gangeticus	10 (Blood) 15 (Tissue)	Indian	G1B-G10B, G1T-G15T
Crocodylus porosus	09(Blood)	Indian	S1B-S9B
Crocodylus palustris	11 (Blood) 04 (Tissue)	Indian	M1B-M11B M1T-M4T
Crocodylus siamensis	06 (Blood)	Indonesia	CSID1-CSID6
Crocodylus niloticus	06 (Blood)	African	CNIA1-CNIA6
Caiman crocodilus	06 (Blood)	South American	CCSA1-CCSA6

Table 2
List of crocodile species and their accession numbers as retrieved from NCBI database.

Species name	Accession number
Tomistoma schlegelii	AJ81045
Osteolaemus tetraspis	AM493868
Alligator mississippiensis	Y13113
Alligator sinensis	AF511507
Melanosuchus niger	EU161678
Paleosuchus palpebrosus	AM493870
Paleosuchus trigonatus	AM493869

2.3. DNA sequencing and data analyses

The PCR products were sequenced using BIG-DYE terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) using the both forward and reverse primers. Sequencing was carried out on Applied Biosystems (Foster City, CA, USA) 3100 Avant Genetic Analyzer according to manufacturer's protocols. The sequences of two amplicons were edited using Bioedit software[12] and the sequences were then used for phylogenetic analysis. The molecular distances were calculated using MEGA 5 software with all available nucleotide substitution models. Two different types of phylogenetic analyses, maximum likelihood (ML) and Bayesian analysis, were performed using PhyML[9] and MrBayes Version 3.1,[13] respectively.

3. Results and discussion

Forensic laboratories often receive degraded samples which fail to yield longer sequences,[11] but long read sequences can be highly beneficial for the flawless identification of species. During molecular study of degraded samples based on mitochondrial DNA (mtDNA) the cytochrome b gene has been found to be a powerful marker for identification of species[21,31,41] and also used in studies of molecular evolution.[18,29] Conversely the level of polymorphism was high in cytochrome b gene compare to 16S rRNA gene, but species specific motifs were higher in 16s rRNA gene.[10] Therefore, we have designed two sets of novel primers within 16S rRNA gene region of mitochondria. The two primer pairs (set I and set II), proposed herein amplified the partial 16S rRNA gene sequences (600bp and

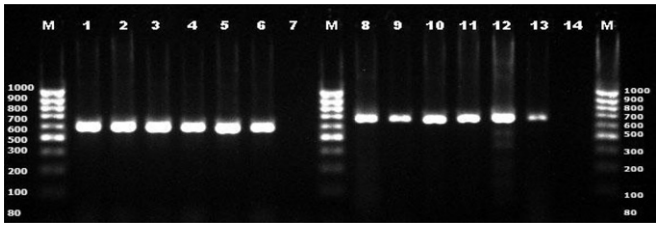

Fig. 1. Two percent agarose gel showing PCR products of six crocodile species, Lanes 1–6 represent 600bp amplicons obtained using primers 16S F1 and 16S R1. Lanes 8–13 represent 690 bp amplicons obtained using primers 16S F2 and 16S R2. Lane M – 1 kb molecular size standards. Lanes 1 and 8 – *G. gangeticus*, Lanes 2 and 9 – *C. porosus*, Lanes 3 and 10 – *C. palustris*, Lanes 4 and 11 – *C. niloticus*, Lanes 5 and 12 – *C. crocodilus*, Lanes 6 and 13 – *C. siamensis*, Lanes 7and 14 – respective negative controls.

Table 3
List of the intraspecies polymorphic sites in six species.

Species (*n* in total)	Position	Nucleotide	*n*
Gavialis gangeticus (25)	10[a]	T	22
		A	3
	31	G	20
		A	5
	443	T	24
		A	1
	983	G	21
		T	4
	1035	G	23
		C	2
Crocodylus porosus (09)	43	T	7
		C	2
	222	G	8
		A	1
	346	T	8
		A	1
	403	A	7
		G	2
	637	A	6
		G	3
	852	C	8
		T	1
Crocodylus palustris (15)	390	G	14
		A	1
	660	G	12
		A	3
	888	C	11
		A	4
	993	G	12
		T	3
Caiman Crocodilus (06)	41	A	4
		C	2
	124	T	5
		C	1
	223	T	4
		A	2
	908	G	5
		C	1
Crocodylus siamensis (06)	31	C	4
		A	2
	105	A	4
		G	2
	211	T	5
		C	1
	702	A	3
		G	3
Crocodylus niloticus (06)	9	G	5
		A	1
	359	G	4
		T	2
	1029	G	3
		A	3

[a] Nucleotide numbering started from the first nucleotide next to the 3′-terminal of forward primer in all species.

690bp respectively) from all six crocodile species (Fig. 1). These sequences were later combined to obtain 1290bp sequence of 16S rRNA gene in six crocodile species i.e. *G. gangeticus*, *C. porosus*, *C. palustris*, *C. siamensis*, *C. niloticus* and *C. crocodilus.* The sequence alignment results show a certain number of polymorphic sites in each species (Table 3), intraspecific polymorphisms are an important deliberation for species identification.[40] To validate the sequence reliability within each species, nucleotide sequences of partial region 16S rRNA within 1290bp were analyzed in multiple individuals of each of the six species, for simplicity, nucleotide position (np) was numbered by calling a site next to 3′-terminal of the forward primer as number 1 in all species. A certain number of polymorphic sites were found in every species, and three of the species i.e. *Crocodylus palustris*, *Caiman Crocodylus* and *Crocodylus siamensis* showed less than four polymorphic sites, similarly *G. gangeticus* showed five polymorphic sites, *C. porosus* showed six polymorphic sites and *C. niloticus* has shown three polymorphic sites. Most of the polymorphic sites were observed in low frequency. Additionally we studied nucleotide diversity by using partial 16S rRNA gene sequences among six crocodile species are summarized (Table 4). The percentage range of sequence diversity was 6.0%–40.7%, and overall mean diversity 24.4%. We compared these values with another forensic marker i.e. Cyt b nucleotide diversity which was 2.4%–22.3% and overall mean diversity 20.1%. These outcome apparently show evaluate to Cyt b gene which is commonly used for species identification,[30,31,42] 16S rRNA is also an important marker for discriminating crocodile species. The sequence data was further used for construction of phylogeny.

Phylogenetic analysis may help in resolving the problems pertaining to sequence consistency and thus may provide an additional accuracy check on the obtained sequences. Crocodilian phylogeny has been already examined in many studies, which have helped to establish higher level phylogenetics relationships between crocodile species.[3,4,16,19,20,24,26,32] Short sequences are not often useful for studying the deep evolutionary divergence with in species[14]; therefore we utilized our newly obtained 1290bp 16S rRNA sequences for the analyses and were aligned with available sequences of other crocodile species in Genbank using MEGA 5.[37] Our phylogeny results obtained from the partial 16S rRNA region analyses supports the monophyly of Indopacific crocodilians (Fig. 2) as reported by previous studies.[23,25] The phylogenetic tree obtained in this study did not deviate from the previously established crocodilian phylogenetic relationships,[24] which in turn verify the accuracy of the sequences obtained by combining two short sequences of 16S rRNA gene. This also adds a further advantage on the utility of these sequences in forensic analyses. Therefore these novel primers can be used to obtain partial 16S rRNA gene sequences from degraded confiscated samples derived from crocodile species. The result of this study shows potential features of these 16S rRNA primers in forensic analysis for crocodiles. Hence, we recommend using these 16s rRNA primers in the forensic identification of endangered crocodiles species, which will help in the effective implementation of wildlife regulations to conserve these keystone species from extinction.

Table 4
Nucleotide sequence diversities in the partial region of 16S rRNA (below diagonal) and Cyt b (above the diagonal)[a] between six crocodile species examined in this study.

	G. gangeticus	*C. porosus*	*C. paulstris*	*C. niloticus*	*C. crocodylus*	*C. siamensis*
G. gangeticus		16.7%	16.5%	17%	19.8%	16.7%
C. porosus	24.2%		6%	7%	22.3%	2%
C. paulstris	20.6%	6%		7%	20.5%	7.5%
C. niloticus	23%	7.8%	7%		22.2%	8%
C. crocodylus	40.7%	22.3%	20.5%	22.2%		22.3%
C. siamensis	22.6%	12.3%	7.8%	8%	22.3%	

[a] Cyt b Nucleotide sequence data was collected from NCBI.

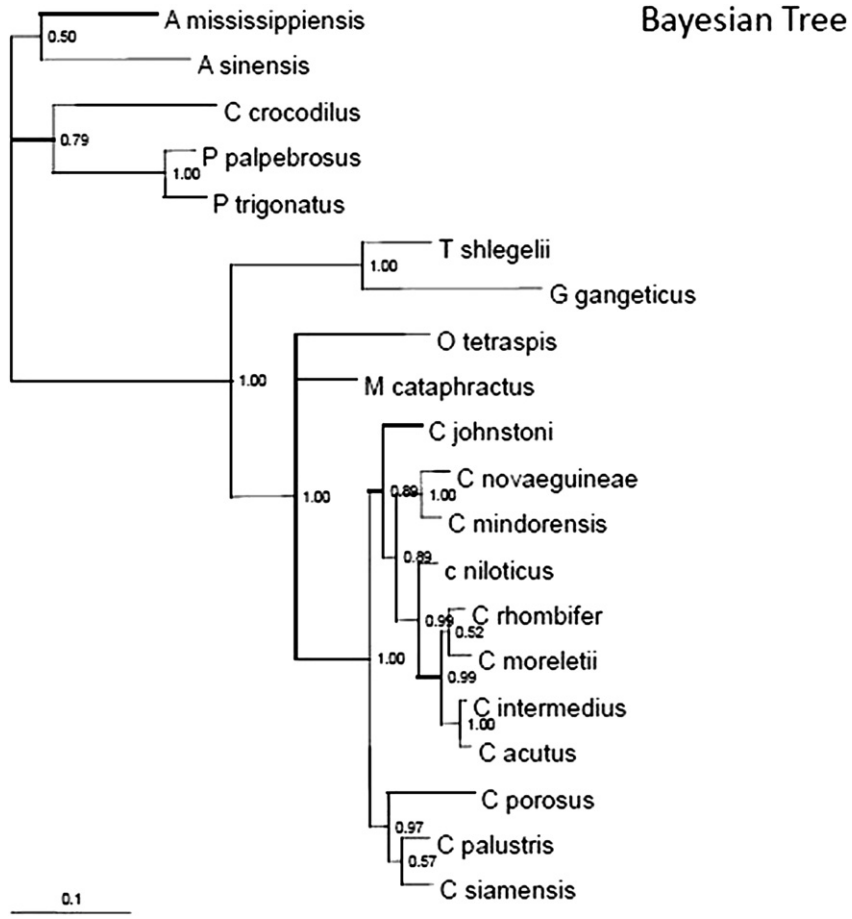

Fig. 2. Bayesian tree with Bayesian probabilities based on 16S rRNA data as obtained using partial sequences.

Ethical approval

Ministry of Environment and Forest, Govt. of India, New Delhi.

Funding

Directorate of Forensic Science Services, Ministry of Home Affairs, Govt. of India, New Delhi, India.

Conflict of interest

All the authors have not transmitted any conflicts of interest.

References

1. An J, Lee MY, Min MS, Lee MH, Lee H. Molecular genetic approach for species identification of mammals and sex determination of birds in forensic case of poaching from South Korea. *Forensic Sci Int* 2006;**167**:59–61.
2. Black WC, Piesman J. Phylogeny of hard and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rRNA sequences. *Proc Natl Acad Sci USA* 1994;**91**: 10034–8.
3. Brochu CA. Phylogenetic relationship and divergence timing of Crocodylus based on morphology and the fossil record. *Copeia* 2000;**2000**:657–73.
4. Brochu CA. Phylogenetic approaches toward crocodilian history. *Annu Rev Planet Sci* 2003;**31**:357–97. http://dx.doi.org/10.1146/annurev.earth.31.100901.141308.
5. Bond JE, Hedin MC, Ramirez MA, Opell BD. Deep molecular divergence in the absence of morphological and ecological change in the Californian coastal dune endemic trapdoor spider Aptostichus simus. *Mol Ecol* 2001;**10**:899–910.
6. Budowle B, Garofano P, Hellman A, et al. Recommendations for animal DNA forensic and identity test. *Int J Legal Med* 2005;**119**:295–302.
7. DeSalle R, Gatesy J, Wheeler W, Grimaldi D. DNA Sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. *Science* 1992;**257**:1933–6.
8. Flook PK, Rowell CHF. The effectiveness of mitochondrial rRNA gene sequences for the reconstruction of the phylogeny of an insect order (Orthoptera). *Mol Phylogenet Evol* 1995;**8**:177–92.
9. Guindon S, Lethiec F, Duroux P, Gascuel O. PHYML online-a web server for fast maximum likelihood phylogenetic inference. *Nucleic Acids Res* 2005;**1**:33.
10. Guha S, Kashyap VK. Development of novel heminested PCR assays based on mitochondrial 16srRNA gene for identification of seven Pecora species. *BMC Genet* 2005;**6**:42. http://dx.doi.org/10.1186/1471-2156-6-42.
11. Hsieh HM, Chiang HL, Tsai LC, Lai SY, Huang NE, Linacre A, et al. Cytocrome b gene for species identification of the conservation of animals. *Forensic Sci Int* 2001;**122**:7–18.
12. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;**41**:95–8.
13. Huelsenbeck JP, Ronquist FR. MRBAYES Bayesian inference phylogenetic tree. *Bioinformatics* 2001;**17**:754–5.
14. Imaizumi K, Akutsu T, Miyasaka S, Yoshino M. Development of species identification tests targeting the 16s ribosomal RNA coding region in mitochondrial DNA. *Int J Leg Med* 2007;**121**:184–91.
15. Irwin DE. Phylogeographic breaks without geographic barriers in gene flow. *Evolution* 2002;**56**:2383–94.
16. Janke A, Gulberg A, Hughes S, Agarwal RK, Arnason V. Mitogenomic analyses place the gharial (Gavialis gangeticus) on crocodile tree and provide pre K/T divergence times for most crocodilians. *J Mol Evol* 2005;**61**:620–6.
17. Kitano T, Umetsu K, Tian W, Osawa M. Two universal primer sets for species identification among vertebrates. *Int J Leg Med* 2007;**121**:423–7.
18. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proc Natl Acad Sci USA* 1989;**86**:6196–200. http://dx.doi.org/10.1073/pnas.86.16.6196.
19. McAliley LR, Willis RE, Ray DA, White PS, Brochu CA, Densmore III LD. Are crocodiles really monophyletic?-evidence for subdivisions from sequence and morphological data. *Mol Phylogenet Evol* 2006;**39**:16–32.
20. Meredith RW, Hekkala ER, Amato G, Gatesy J. Aphylogenetic hypothesis for *Crocodylus* (Crocodylia) based on mitochondrial DNA: evidence for a trans-Atlantic voyage from Africa to the New World. *Mol Phylogenet Evol* 2011;**60**:183–91.
21. Meganathan PR, Dubey B, Haque I. Molecular identification of crocodile species using novel primers for forensic analysis. *Conserv Genet* 2009;**10**:767–70.
22. Meganathan PR, Dubey B, Haque I. Molecular identification of Indian crocodile species: PCR-RFLP method for forensic authentication. *J Forensic Sci* 2009;**54**: 1042–5.
23. Meganathan PR, Dubey B, Jogayya KN, Whitaker N, Haque I. A novel multiplex PCRassay for the identification of Indian crocodiles. *Mol Ecol Resour* 2010. http://dx.doi.org/10.1111/j.1755-0998.2010.02834.x.

24. Meganathan PR, Dubey B, Batzer MA, Ray DA, Haque I. Molecular phylogenetic analyses of genus Crocodylus (Eusuchia, Crocodylia, Crocodylidae) and the taxonomic position of Crocodylus porosus. *Mol Phylogenet Evol* 2010b;**57**:393–402.
25. Meganathan PR, Dubey B, Jogayya KN, Haque I. Validation of a multiplex PCR assay for the forensic identification of Indian crocodiles. *J Forensic Sci* 2011a;**56**:1241–4.
26. Meganathan PR, Dubey B, Batzer MA, Ray DA, Haque I. Complete mitochondrial genome sequences of three Crocodylus species and their comparison within the Order Crocodylia. *Gene* 2011b;**478**:35–41.
27. Meganathan PR, Dubey B, Jogayya KN, Haque I. Identification of Indian crocodile species through DNA barcodes. *J Forensic Sci*, in press.
28. Miles LJ, Lance SL, Isberg SR, Moran C, Glenn TC. Cross- species amplification of microsatellites in crocodilians: assessment and applications for the future. *Conserv Genet* 2008. http://dx.doi.org/10.1007/s10592-008-9601-6.
29. Montgelard C, Catzeflis FM, Douzery E. Phylogenetic relationships of artidactyls and cetaceans as deduced from comparison of cytochrome b and 12S rRNA mitochondrial sequences. *Mol Biol Evol* 1997;**14**:555–9.
30. Murray BW, McClymont RA, Strobeck C. Forensic identification of ungulate species using restriction digests of PCR amplified mitochondrial DNA. *J Forensic Sci* 1995;**40**:943–51.
31. Parson W, Pegoraro K, Niederstatter H, Foger M, Stein lechner M. Species identification by means of the cytochrome b gene. *Int J Leg Med* 2000;**114**: 23–8.
32. Roos J, Agarwal RK, Janke A. Extended mitogenomic phylogenetic analyses yield new insight into crocodilian evolution and their survival of Cretaceous–Tertiary boundary. *Mol Phylogenet Evol* 2007;**45**:663–73. http://dx.doi.org/10.1016/j.ympev.2007.06.018.
33. Sambrook J, Russel DW. *Molecular cloning: a laboratory manual*. New York: Cold spring Harbor Laboratory Press; 2001.
34. Santiapillai C, de Silva M. Status, distribution and conservation of crocodiles in Sri Lanka. *Biol Conserv* 2001;**97**:305–18. http://dx.doi.org/10.1016/S0006-3207(00)00126-9.
35. Stuart BL, Hayens B, Manh BH, Platt SG. Status of crocodiles in the U Minh Thuong Nature Reserve Southern Vietnamm. *Pac Conserve Biol* 2002;**8**:62–5.
36. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;**22**: 4673–80. http://dx.doi.org/10.1093/nar/22.22.4673.
37. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011. http://dx.doi.org/10.1093/molbev/msr121.
38. Sk Verma, Singh L. Novel universal primers establish identity of an enormous number of animal species fro forensic applications. *Mol Ecol Notes* 2003;**3**:28–31. http://dx.doi.org/10.1046/j.1471-8286.2003.00340.x.
39. Verma SK, Prasad K, Nagesh N, Sultana M, Singh L. Was elusive carnivore a panther? DNA typing of faeces reveals the mystery. *Forensic Sci Int* 2003;**137**: 16–20. http://dx.doi.org/10.1016/S0379-0738(03)00277-9.
40. Waits LP, Paetkau D. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *J Wildl Manage* 2005;**69**:1419–33.
41. Wu H, Wan QH, Fang SG, Zhang SY. Application of mitochondrial DNA sequence analysis in the forensic identification of Chinese sika deer subspecies. *Forensic Sci Int* 2005;**148**:101–5.
42. Zehner R, Zimmermann S, Mebs D. RFLP and sequence analysis of the cytochrome b gene of selected animals and man: methodology and forensic application. *Int J Leg Med* 1998;**111**:323–7.